HUMANIZATION OF MURINE ANTIBODY

Cross-Reference to Related Applications

This application is a continuation of copending Continued Prosecution Application Serial No. 08/986,016 filed on November 17, 2000, which is a continuation of U. S. Patent Application Serial No. 08/986,016 filed on December 5, 1997.

Governmental Rights

This invention was made with government support under Contract No. AI 37470 by the National Institutes of Health. The government has certain rights in the invention.

Technical Field

This invention relates to humanization of murine antibodies.

Background of the Invention

Antibodies typically comprise two heavy chains linked together by disulfide bonds and two light chains linked to a respective heavy chain by a disulfide bond. Beginning at one end of each heavy chain there is a variable domain followed by several constant domains. Similarly, each light chain has a variable domain at one end, but only a single constant domain at its other end. There are two types of light chain, which are termed lambda (λ) and kappa (κ) chains. No functional difference has been found between antibodies having λ or κ light chains. The ratio of the two types of light chain varies from species to species, however. In mice, the κ:λ ratio is 20:1, whereas in humans it is 2:1.

The variable domains of the light and heavy chains are aligned, as are the constant domain of the light chain and the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to antigen.

It is the variable domains that form the antigen binding site of antibodies. The general structure of each light and heavy chain domain comprises a framework of four regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The four framework regions employ a beta-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

While cell surface antigens of tumor cells are the traditional targets for antibody-guided cancer therapy, one of the major limitations for the therapy of solid tumors is the low accessibility of tumor antigens to antibodies circulating in the blood stream. The dense packing of tumor cells and the elevated interstitial pressure in the tumor core present formidable physical barriers.

A solution to the problem of poor penetration of antibodies into solid tumors would be to attack the endothelial cells lining the blood vessels of the tumor rather than the tumor cells themselves. While it may be difficult to target the mature tumor vasculature specifically, i.e., without destroying healthy tissue, promising strategies aim at the inhibition of neovascularization.

Neovascularization, also termed angiogenesis, is induced by cytokines that are secreted from tumor cells and depends on vascular cell migration and invasion, processes regulated by cell adhesion molecules (CAM) and protease. These molecules are currently considered potential targets for angiogenic inhibitors. In this regard, the vascular integrin $\alpha_v\beta_3$ has recently been identified as a marker of angiogenic blood vessels. *See* Brooks, P.C., et al. (1994), REQUIREMENT OF INTEGRIN $\alpha_v\beta_3$ FOR ANGIOGENESIS, *Science* **264**, 569-571. Moreover, it was shown that the mouse monoclonal antibody (Mab) LM609 directed to integrin $\alpha_v\beta_3$ was able to suppress angiogenesis, indicating than integrin $\alpha_v\beta_3$ has a critical role in angiogenesis.

It has been further demonstrated that LM609 selectively promotes apoptosis of vascular cells that have been stimulated to undergo angiogenesis. *See* Brooks, P.C., et al. (1994), INTEGRIN $\alpha_v\beta_3$ ANTAGONISTS PROMOTE TUMOR REGRESSION BY INDUCING APOPTOSIS OF ANGIOGENIC BLOOD VESSELS, *Cell* **79**, 1157-1164. These findings suggest that integrin $\alpha_v\beta_3$ may be a target and LM609 a tool for cancer diagnosis and therapy.

Indeed, LM609 not only prevented the growth of histologically distinct human tumors implanted on the chorioallantoic membranes of chicken embryos, but also induced their regression. *See, Cell* **79**, 1157-1164. Using a more clinically relevant model of tumor growth, it was found that LM609 blocked human breast cancer growth in a SCID mouse/human chimeric model. Importantly, not only did LM609 block tumor growth, but it also inhibited metastasis of the breast carcinomas examined. *See* Brooks, et al. (1995) ANTI-INTEGRIN $\alpha_v\beta_3$ BLOCKS HUMAN BREAST CANCER GROWTH AND ANGIOGENESIS IN HUMAN SKIN, *J.Clin. Invest.* **96**, 1815-1822.

The Brooks et al. results are consistent with previous studies that have suggested that angiogenesis contributes to the metastatic spread of breast tumor cells. *See* Weidner, N., et al. (1991) TUMOR ANGIOGENESIS AND METASTASIS: CORRELATION IN INVASIVE BREAST CARCINOMA, *N.Engl. J.Med.* **324**, 1-8; and Weidner, N., et al. (1992) TUMOR ANGIOGENESIS: A NEW SIGNIFICANT AND INDEPENDENT PROGNOSTIC INDICATOR IN EARLY-STAGE BREAST CARCINOMA, *J.Natl. Cancer Inst.* **84**, 1875-1887.

Within the last few years evidence has been presented that two cytokine-dependent pathways of angiogenesis exist and that these are defined by their dependency on distinct vascular integrins. *See* Friedlander, M., et al. (1995) DEFINITION OF TWO ANGIOGENIC PATHWAYS BY DISTINCT AV INTEGRINS, *Science* **270**, 1500-1502. The results of the Friedlander et al. studies show that anti-$\alpha_v\beta_3$ antibody LM609 blocked angiogenesis in response to bFGF and TNFα, yet have little effect on angiogenesis induced by VEGF, TGFα, or phorbol ester PMA. In contrast, the anti-$\alpha_v\beta_5$ antibody P1F6 blocks angiogenesis induced by VEGF, TGFα, and phorbol ester PMA, while having minimal effects on that induced by bFGF or TNFα.

It is conceivable, thus, that tumors showing less susceptibility to anti-$\alpha_v\beta_3$ antibodies might secrete cytokines that promote angiogenesis in an $\alpha_v\beta_5$-dependent manner. Taken together, both anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ antibodies are promising tools for diagnosis and therapy of cancer.

Mouse monoclonal antibodies such as LM609, however, are highly immunogenic in humans, thus limiting their potential use for cancer therapy, especially when repeated administration is necessary. To reduce the immunogenicity of mouse monoclonal antibodies, chimeric

monoclonal antibodies were generated, with the variable Ig domains of a mouse monoclonal antibody being fused to human constant Ig domains. *See* Morrison, S.L., et al. (1984) CHIMERIC HUMAN ANTIBODY MOLECULES; MOUSE ANTIGEN-BINDING DOMAINS WITH HUMAN CONSTANT REGION DOMAINS, *Proc. Natl. Acad. Sci. USA* **81**, 6841-6855; and, Boulianne, G.L., et al. (1984) PRODUCTION OF A FUNCTIONAL CHIMAERIC MOUSE/HUMAN ANTIBODY, *Nature* **312**, 643-646. This process is commonly referred to as "humanization" of an antibody.

In general, the chimeric monoclonal antibodies retain the binding specificity of the mouse monoclonal antibody and exhibit improved interactions with human effector cells. This results in an improved antibody-dependent cellular cytotoxicity which is presumed to be one of the ways of eliminating tumor cells using monoclonal antibodies. *See* Morrison, S.L. (1992) *IN VITRO* ANTIBODIES: STRATEGIES FOR PRODUCTION AND APPLICATION, *Ann. Rev. Immunol.* **10**, 239-265. Though some chimeric monoclonal antibodies have proved less immunogenic in humans, the mouse variable Ig domains can still lead to a significant human anti-mouse response. *See* Bruggemann, M., et al. (1989) THE IMMUNOGENICITY OF CHIMERIC ANTIBODIES, *J.Exp. Med.* **170**, 2153-2157. Therefore, for therapeutic purposes it may be necessary to fully humanize a murine monoclonal antibody by altering both the variable and the constant Ig domains.

Full humanization is feasible by introducing the six CDRs from the mouse heavy and light chain variable Ig domains into the appropriate framework regions of human variable Ig domains. This CDR grafting technique (Riechmann, L., et al. (1988) RESHAPING HUMAN ANTIBODIES FOR THERAPY, *Nature* **332**, 323) takes advantage of the conserved structure of the variable Ig domains, with the four framework regions (FR1-FR4) serving as a

scaffold to support the CDR loops which are the primary contacts with antigen. U.S. Patent No. 5,502,167 to Waldmann, et al. discloses a "humanised antibody" having the CDR loops LCDR1 through LCDR3 and HCDR1 through HCDR3 from YTH 655(5)6, a rat IgG2b monoclonal antibody, grafted onto a human T cell antibody.

A drawback, however, of the CDR grafting technique is the fact that amino acids of the framework regions can contribute to antigen binding, as well as amino acids of the CDR loops can influence the association of the two variable Ig domains. To maintain the affinity of the humanized monoclonal antibody, the CDR grafting technique relies on the proper choice of the human framework regions and site-directed mutagenesis of single amino acids aided by computer modeling of the antigen binding site (e.g., Co, M.S., et al. (1994) A HUMANIZED ANTIBODY SPECIFIC FOR THE PLATELET INTEGRIN gpIIb/IIa, *J.Immunol.* **152**, 2968-2976). A number of successful humanizations of mouse monoclonal antibodies by rational design have been reported. Among them are several monoclonal antibodies that are directed to human integrins and have potential clinical application. *See, J.Immunol.* **152**, 2968-2976; Hsiao, K.C., et al. (1994) HUMANIZATION OF 60.3, AN ANTI-CD18 ANTIBODY; IMPORTANCE OF THE L2 LOOP, *Protein Eng.* **7**, 815-822; and, Poul, M.A., et al. (1995) INHIBITION OF T CELL ACTIVATION WITH A HUMANIZED ANTI-BETA 1 INTEGRIN CHAIN mAb, *Mol. Immunol.* **32**, 101-116.

Human immunoglobulin transgenic mice provide a promising alternative to the humanization of mouse monoclonal antibodies. *See, e.g.,* Fishwild, D.M., et al. (1996) HIGH-AVIDITY HUMAN IgGK MONOCLONAL ANTIBODIES FROM A NOVEL STRAIN OF MINILOCUS TRANSGENIC MICE, *Nature Biotechnology* **14**, 845-851. In response to immunization,

these mice express human monoclonal antibodies, which can be accessed by conventional hybridoma technology.

Rational design strategies in protein engineering have been challenged by *in vitro* selection strategies that are mainly based on phage display libraries. *See* Clackson, T., and Wells, J.A. (1994) *IN VITRO* SELECTION FROM PROTEIN AND PEPTIDE LIBRARIES, *TIBTECH* **12**, 173-184. In particular, *in vitro* selection and evolution of antibodies derived from phage display libraries has become a powerful tool. *See* Burton, D.R., and Barbas III, C.F. (1994) HUMAN ANTIBODIES FROM COMBINATORIAL LIBRARIES, *Adv. Immunol.* **57**, 191-280; and, Winter, G., et al. (1994) MAKING ANTIBODIES BY PHAGE DISPLAY TECHNOLOGY, *Annu. Rev. Immunol.* **12**, 433-455.

The development of technologies for making repertoires of human antibody genes, and the display of the encoded antibody fragments on the surface of filamentous bacteriophage, has provided a means for making human antibodies directly. The antibodies produced by phage technology are produced as antigen binding fragments—usually Fv or Fab fragments—in bacteria and thus lack effector functions. Effector functions can be introduced by one of two strategies: The fragments can be engineered either into complete antibodies for expression in mammalian cells, or into bispecific antibody fragments with a second binding site capable of triggering an effector function.

Typically, the Fd fragment (V_H-C_H1) and light chain (V_L-C_L) of antibodies are separately cloned by PCR and recombined randomly in combinatorial phage display libraries, which can then be selected for binding to a particular antigen. The Fab fragments are expressed on the phage surface, i.e., physically linked to the genes that encode them. Thus, selection of Fab by antigen

binding co-selects for the Fab encoding sequences, which can be amplified subsequently. By several rounds of antigen binding and reamplification, a procedure termed panning, Fab specific for the antigen are enriched and finally isolated.

In 1994, an approach for the humanization of antibodies, called "guided selection", was described. Guided selection utilizes the power of the phage display technique for the humanization of mouse monoclonal antibody. *See* Jespers, L.S., et al. (1994) GUIDING THE SELECTION OF HUMAN ANTIBODIES FROM PHAGE DISPLAY REPERTOIRES TO A SINGLE EPITOPE OF AN ANTIGEN, *Bio/Technology* **12**, 899-903. For this, the Fd fragment of the mouse monoclonal antibody can be displayed in combination with a human light chain library, and the resulting hybrid Fab library may then be selected with antigen. The mouse Fd fragment thereby provides a template to guide the selection.

Subsequently, the selected human light chains are combined with a human Fd fragment library. Selection of the resulting library yields entirely human Fab.

For the full humanization of murine monoclonal antibodies, the present invention uses a unique combination of CDR grafting and guided selection. The anti-integrin antibody generated is useful for cancer diagnosis and therapy.

Summary of the Invention

Humanization of a mouse monoclonal antibody is achieved by a combination of guided selection and CDR grafting. The term "humanized" as used herein and in the appended claims means that at least one chain of a

mouse monoclonal antibody includes a region of a human monoclonal antibody.

A humanized mouse monoclonal antibody is produced by constructing a library of human antibody heavy chains or light chains in which each such chain includes a variable domain and has at least one complementarity determining region (CDR) amino acid sequence which is that of a corresponding mouse heavy or light antibody chain, and then combining the library so constructed with a complementary chain from an antibody which binds a preselected antigen. In this manner, the complementary chain together with a human chain present in the constructed library forms a heavy and light chain pair in a resulting library of humanized chain pairs. Thereafter a particular humanized heavy and light chain pair is selected from the humanized pair library using the aforementioned complementary chain.

In a particular embodiment, a mouse monoclonal antibody can be humanized by constructing a human light chain library in which each light chain includes at least the variable domain thereof and at least one CDR amino acid sequence of a mouse light chain, and a human heavy chain library in which each such heavy chain includes at least the variable domain thereof and at least one CDR amino acid sequence of a mouse heavy chain. The heavy chain usually is no more than about 200 amino acid residues in size.

A human light chain having a mouse CDR is selected from the constructed human light chain library using a heavy chain from an antibody which binds a preselected antigen. The constructed heavy chain library is combined with the selected human light having a mouse CDR to produce a humanized library of heavy and light chain pairs, each containing at least one mouse

CDR. Thereafter, a heavy and light chain pair with mouse CDR is selected from the aforesaid humanized library using the selected human light chain with mouse CDR. The sequence of aforementioned library construction is not critical.

Preferably, only the light chain complementarity determining region three (LCDR3) loop of the monoclonal antibody is grafted onto the human light chain. Similarly, it is preferable that only the HCDR3 loop be grafted onto the human heavy chain (HC) fragment. The selection of either the human light chain or human heavy chain having the grafted mouse CDR is preferably made by using a chimeric mouse/human complementary chain as a template.

In CDR grafting onto a human light chain, the human light chain is cloned, then the clones are randomly recombined to form a library such as a combinatorial phage display library. The same method can be followed for grafting onto the human heavy chain.

Brief Description of the Drawings

FIGURE 1 is a schematic illustration showing the sequence of the steps in the combined CDR grafting technique and guided selection technique to form the humanized Fab fragment.

FIGURES 2a and 2b show the amino acid sequences of V_λ and V_κ, respectively, of mouse monoclonal antibody LM609. The N-terminal two amino acids (Leu)(Glu) of V_λ and (Glu)(Leu) of V_κ encoded by the vector cloning sites CTCGAG (Xhol) and GAGCTC (Sacl), respectively, are artificial. The CDR loops are underlined.

FIGURE 3a through FIGURE 3e show the amino acid sequence alignment of mouse LM609 V_κ (top full line

of each sequence grouping) and six selected human V_κ's (#1-6). Framework regions (FR1-3) and CDR (CDR1-2) loops are separated. Lines (-) indicate identical amino acids. Note that due to the LCDR3 grafting parts of FR3, entire CDR3 and entire FR4 are identical in mouse LM609 V_κ and the selected human V_κ's. Therefore, these two sequences are not shown.

FIGURE 4 shows a comparison of three selected human fragment sequences and four unselected human fragment sequences to the original sequences of the mouse LCDR1 and LCDR2 loops.

FIGURE 5a through FIGURE 5f are line graphs which show the binding of human integrin $\alpha_V\beta_3$ on the cell surface by humanized LM609 clones 2, 4, 7, 11, 24, and control antibody, respectively. Line A indicates untransfected CS-1 hamster cells; Line B indicates human β_5 cDNA transfected CS-1 hamster cells (essentially the same line as line A in FIGURE 5f); and line C indicates human β_3 cDNA transfected CS-1 hamster cells.

FIGURE 6 is a bar graph showing the cross-reactivity of the LM609 antibody, and clones 2, 4, 7, 11, 24, and control antibody, respectively. Columns represent the mean of triplicates, with the left columns indicating binding to human integrin $\alpha_V\beta_3$, the central columns indicating binding to human integrin $\alpha_{IIb}\beta_3$, and the right columns indicating background binding. Error bars indicate standard deviations.

FIGURE 7 is a schematic illustration of a stretch of four amino acids in a light chain complementarity determining region three (LCDR3) and a heavy chain complementarity determining region three (HCDR3) being optimized.

FIGURES 8a and 8b are fragmented illustrations of the V_L amino acid sequences of a mouse antibody

compared to the amino acid sequences of five versions of humanized clones represented by group letters A (clones 10, 11, and 37), and B (clones 7, 8, and 22), C (clones 4, 31, and 36), D (clones 24, 34, 35, and 40), and E (clone 2) which are combined.

FIGURES 8c through 8e are fragmented illustrations of the V_H amino acid sequences of a mouse antibody compared to the amino acid sequences of five versions of humanized clones represented by group letters A (clones 10, 11, and 37), B (clones 7, 8, and 22), C (clones 4, 31, and 36), D (clones 24, 34, 35, and 40), and E (clone 2).

Description of a Preferred Embodiment

While the present invention is susceptible to embodiments in many different forms, a preferred embodiment of the invention is described below. It should be understood, however, that the present disclosure is to be considered as an exemplification of the principles of the invention and is not intended to limit the invention to the specific embodiments illustrated.

cDNA Cloning of mouse monoclonal antibody LM609

Beginning with a LM609 hybridoma cell line (ATCC Designation HB 9537), total RNA was prepared from 10^8 LM609 hybridoma cells using an RNA Isolation Kit (Stratagene, La Jolla, CA). Reverse transcription and polymerase chain reaction (PCR) amplification of Fd fragment and κ chain encoding sequences were performed essentially as described in "Combinatorial immunoglobulin libraries in phage 1", (*Methods* **2**, 119 (1991)) by A.S. Kang, et al.

Fd fragment and κ chain encoding PCR products were cut with XhoI/SpeI and SacI/XbaI, respectively, and

ligated sequentially into the appropriately digested phagemid vector pComb3H. The ligation products were introduced into *E.coli* strain XL1-Blue by electrotransformation and subsequent steps were as described in "Assembly of combinatorial antibody libraries on phage surfaces: the gene III site", (*Proc. Natl. Acad. Sci. USA* **88**, 7978-7982) by C.F. Barbas III, et al., to produce phage displaying Fab on their surface. Phage were selected by panning against immobilized integrin $\alpha_v\beta_3$. After two panning rounds single clones were analyzed for LM609 Fab expression. Supernatant from IPTG-induced cultures was tested for binding to immobilized integrin $\alpha_v\beta_3$ by enzyme-linked immunosorbent assay (ELISA) using goat anti-mouse $F(ab')_2$ conjugated to alkaline phosphatase as a secondary antibody. The sequence of each Fd fragment and each κ chain encoding sequence of positive clones was determined by DNA sequencing.

Amplification of human light chain and Fd fragment sequences

Total RNA was prepared from the bone marrow of five donors (Poietic Technologies; Germantown, MD) using TRI Reagent (Molecular Research Center; Cincinnati, OH) and was further purified by lithium chloride precipitation. *See* Sambrook, J., et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. First-strand cDNA was synthesized using the 'SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis' kit with oligo (dT) priming (Life Technologies; Gaithersburg, MD). The five generated first-strand cDNAs were subjected to separate PCR amplifications.

V_κ sequences of each of the first-strand cDNAs were amplified in eight separate reactions by combining four sense and two antisense primers (see list below). V_λ sequences were amplified in nine separate reactions using nine sense and one antisense primer (see list below). The V_λ and V_κ amino acid sequences, including the underlining of the CDR loops, are shown in FIGURES 2a and 2b, respectively (See also, SEQ ID NO:44 and SEQ ID NO:45, respectively).

V_H sequences (See SEQ ID NO:56) were amplified in four reactions using four sense and one antisense primer (see list below). All amplifications were performed under standard PCR conditions using Taq polymerase (Pharmacia; Uppsala, Sweden). While the sense primers hybridize to sequences that encode the N-terminal amino acids of the various V_κ, V_λ, and V_H families, the antisense primers hybridize to a sequence that encodes the C-terminal amino acids of FR3 of V_κ, V_λ, or V_H, respectively, which are highly conserved.

The list of primers used for the amplification of human antibody sequences include:

V_κ sense primers:

HSCK1 - F	SEQ ID NO:3	HSCLam6	SEQ ID NO:14
HSCK24 - F	SEQ ID NO:4	HSCLam70	SEQ ID NO:15
HSCK3 - F	SEQ ID NO:5	HSCLam78	SEQ ID NO:16
HSCK5 - 5	SEQ ID NO:6	HSCLam9	SEQ ID NO:17
V_κ antisense primers:		V_λ antisense primer:	
BKFR3UN	SEQ ID NO:7	BLFR3UN	SEQ ID NO:18
BK2FR3UN	SEQ ID NO:8	V_H sense primers:	
V_λ sense primers:		HFVH1-F	SEQ ID NO:19
HSCLam1a	SEQ ID NO:9	HFVH2-F	SEQ ID NO:20
HSCLam1b	SEQ ID NO:10	HFVH35-F	SEQ ID NO:21
HSCLam2	SEQ ID NO:11	HFVH4-F	SEQ ID NO:22
HSCLam3	SEQ ID NO:12	V_H antisense primers:	
HSCLam4	SEQ ID NO:13	BFR3UN	SEQ ID NO:23

Construction of a chimeric mouse/human Fd fragment by fusing V_H of LM609 to human C_H1

The phagemid vector pComb3H containing the LM609 Fab sequences was used as a template for amplification of the sequence encoding the N-terminal FR1 through FR3 fragment of LM609's V_H by the PCR primer pair PELSEQ (SEQ ID NO:24) / BFR3UN (SEQ ID NO:25). The sense primer PELSEQ hybridizes to the pe1B leader sequence upstream of the Fd fragment encoding sequence in pComb3H. The antisense primer BFR3UN hybridizes to a sequence that encodes eight C-terminal amino acids of FR3 of V_H, which are highly conserved (SEQ ID NO:26), and differ in one amino acid from the corresponding amino acid sequence of LM609's V_H (SEQ ID NO:27).

By overlap extension PCR (*See* McArn Horton, R., and Readington Pease, L. (1991) RECOMBINATION AND MUTAGENESIS OF DNA SEQUENCES USING PCR IN *DIRECTED MUTAGENESIS: A*

PRACTICAL APPROACH, ed. M.J. McPherson, IRL Press, Oxford, UK, pp. 217-247), the PELSEQ/BFR3UN product was fused to a PCR fragment encoding the HCDR3 (SEQ ID NO:1) of LM609 coupled to FR4 of V_H and the entire C_H1 domain of the human anti-gp120 antibody b8. This fragment had been amplified by the PCR primer pair CR501 (SEQ ID NO:28) / CR301 (SEQ ID NO:29). The sense primer CR501 encodes a synthetic link of the nine C-terminal amino acids of FR3, the eight amino acids forming the HCDR3 (SEQ ID NO:1) of LM609, and the six N-terminal amino acids of FR4 of b8. The FR4 of b8 is a preferred choice here because it is identical to FR4 of LM609's V_H with the exception of the C-terminal amino acid, which is A for LM609 and S for b8. The 24-bp overlap of CR501 and BFR3UN allowed to fuse the corresponding PCT products by overlap extension PCR.

The sense primer CR301 hybridizes to a sequence that encodes the C terminus of C_H1 and introduces a SpeI site that allows the PCR product to link to the gene III ORF on pComb3H. The product of the overlap extension PCR was cut with XhoI/SpeI, ligated into the appropriately digested phagemid vector pComb3H, and the correct sequence was confirmed by DNA sequencing.

Substitution of the LM609 light chain by a human light chain that contains the LCDR3 of LM609

Using overlap extension PCR, the amplified human sequences encoding the N-terminal FR1 through FR3 fragment of V_κ and V_λ were fused to PCR fragments encoding the LCDR3 (SEQ ID NO:2) of LM609 coupled to FR4 of human V_κ or V_λ and the human C_κ or C_λ domain. Two κ fragments were generated by the PCR primer pairs CR503 (SEQ ID NO:30) / T7B (SEQ ID NO:31) and CR508 (SEQ ID

NO:32) / T7B using the sequence of the anti-gp120 antibody b11 in pComb3 as a template.

The sense primers CR503 and CR508 encode a synthetic link of eight C-terminal amino acids of FR3 of human V_κ (SEQ ID NO:33 or SEQ ID NO:34), the nine amino acids forming the LCDR3 (SEQ ID NO:2) of LM609, and the seven N-terminal amino acids of FR4 of b11. FR4 of b11 is the preferred choice because it is identical to FR4 of LM609's V_κ with the exception of the third N-terminal and C-terminal amino acid, which are G and T in LM609 versus Q and A in b11. The 23-bp overlap of CR503 with BKFR3UN and CR508 with BK2FR3UN allowed to fuse the corresponding PCR products by overlap extension PCR.

The backward primer T7B hybridizes to a pComb3 sequence downstream of the light chain encoding sequence. A λ fragment was generated by the PCR primer pair CR510 (SEQ ID NO:35) / CLext (SEQ ID NO:36) using CLext primed first strand cDNA from human bone marrow as a template.

The sense primer CR510 encodes a synthetic link of seven C-terminal amino acids of FR3 of human V_λ (SEQ ID NO:37), the nine amino acids forming the LCDR3 of LM609, and the seven N-terminal amino acids of FR4 of human V_λ (SEQ ID NO:38). The 21-bp overlap of CR510 with BLFR3UN allowed to fuse the corresponding PCR products by overlap extension PCR. The backward primer CLext hybridizes to the 3' end of the human C_λ encoding sequence and introduces a Xbai site.

The generated light chain encoding sequences were cut with SacI/XbaI and ligated into the appropriately digested phagemid vector pComb3H that contained the chimeric mouse/human Fd fragment. Electrotransformation of the ligation products into *E.coli* strain ER 2537 (New England Biolabs; Beverly, MA)

resulted in a light chain library consisting of 1.5 x 10^8 independent transformants. DNA sequencing revealed the correct assembly of the fused fragments.

Four rounds of panning against immobilized human integrin $\alpha_v\beta_3$ were carried out essentially as described in "High-affinity self-reactive human antibodies by design and selection: targeting the integrin ligand binding site", (*Proc. Natl. Acad. Sci. USA* **90**, 10003-10007 (1993)) by C.F. Barbas, III, et al. using 200 ng protein in 25 µl metal buffer (25 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM $MnCl_2$) for coating, 0.05 percent Tween 20 in TBS for washing, and 10 mg/ml trypsin in TBS for elution. The washing steps were increased from 5 in the first round, to 10 in the second round and 15 in the third and fourth rounds. The output phage pool of each round was monitored by phage ELISA.

After the fourth round phage were produced from single clones and tested for binding to immobilized integrin $\alpha_v\beta_3$ by phage ELISA. Light chain encoding sequences of positive clones were analyzed by DNA sequencing using the primer OMPSEQ (SEQ ID NO:39) that hybridizes to the ompA leader sequence upstream of the light chain encoding sequence in pComb3H.

Substitution of the LM609 Fd fragment by a human Fd fragment that contains the heavy complementarity determining region three (HCDR3) of LM609

Three PCR fragments were fused in one step by overlap extension PCR. Using the selected phagemids from the light chain panning as a template, Fragment 1 was amplified with the PCR primer pair RSC-F (SEQ ID NO:40) / lead-B (SEQ ID NO:41). While the sense primer RSC-F hybridizes to a sequence upstream of the light

chain encoding sequence, the antisense primer lead-B hybridizes to a sequence upstream of the Fd fragment encoding sequence. The amplified human sequences encoding the FR1 through FR3 fragment of VH (see above) were used as fragment 2.

Fragment 3 was amplified with the PCR primer pair CR501 / HIgG1-B (SEQ ID 42) using the hybrid mouse/human Fd fragment (see above) as a template. The antisense primer HIgG1-B hybridizes to the 3' end of the C_H1 encoding sequence. Using the 21-bp overlap of lead-B with the HFVH-F primers and the 24-bp overlap of BFR3UN with CR501, the three fragments were fused and amplified with the PCR primer pair RSC-F / RSC-B (SEQ ID NO:43). The antisense primer RSC-B overlaps with HIgG1-B. RSC-F and RSC-B introduce two asymmetric SfiI sites.

To maintain high complexity, separate PCR reactions were performed for each selected phagemid from the light chain panning (Fragment 1) and for each of the five V_H fragment pools derived from the five first strand cDNA sources (Fragment 2). The generated fragments encoding the selected human light chains linked to human Fd fragments were cut with SfiI and ligated into the appropriately digested phagemid vector pComb3H generating a library of 3×10^7 independent transformants.

DNA sequencing revealed the correct assembly of the fused DNA fragments. Four rounds of panning against immobilized human integrin $\alpha_v\beta_3$ were carried out exactly as described for the light chain panning. The output phage pool of each round was monitored by phage ELISA. After the fourth round, light chain and Fd fragment encoding sequences were isolated from the selected phagemids by SfiI digestion and subcloned into the compatible expression vector pPhoA-H6HA.

Lysates of individual clones grown in phosphate-deprived medium were analyzed for binding to immobilized integrin $\alpha_V\beta_3$ by ELISA using goat anti-human $F(ab')_2$ conjugated to alkaline phosphatase (Pierce) as secondary antibody. Light chain and Fd fragment encoding sequences of positive clones were analyzed by DNA sequencing using the primers OMPSEQ and PELSEQ, respectively.

Results

cDNA Cloning of LM609

Starting from LM609 expressing hybridoma cells, cDNAs encoding λ chain Fd fragments and entire κ chains were cloned by PCR. The PCR products were cloned into the phagemid pComb3H, which is derived from pComb3, and engineered to facilitate the expression of Fab on the surface of M13 filamentous phage. Phage displaying LM609 Fab were selected by panning against integrin $\alpha_V\beta_3$ and the corresponding cDNA sequences were determined. Soluble LM609 Fab purified from *E.coli* was analyzed and found to bind specifically to integrin $\alpha_V\beta_3$ by ELISA.

The approach for the sequential humanization of LM609 by a combination of guided selection and CDR grafting is illustrated in FIGURE 1. For the human light chain selection, the mouse Fd fragment is substituted by a chimeric Fd fragment composed of mouse V_H linked to human C_H1 to stabilize the hybrid Fab of the first selection step by the interaction of two matching human constant domains, C_κ and C_H1. A stabilization of the hybrid Fab also stabilizes the antigen binding site.

The guided selection is started by substituting the κ light chain of LM609 with a human κ

and λ light chain library that contained the grafted LCDR3 loop of LM609. The corresponding phage libraries displaying hybrid Fab are then selected by four rounds of panning against immobilized integrin $\alpha_V\beta_3$. Though the output number does not increase from round to round, analysis of the output phage pool from each round for binding to integrin $\alpha_V\beta_3$ by phage ELISA reveals an increasing signal. After the fourth round, phage are produced from clones and tested for binding to integrin $\alpha_V\beta_3$ by phage ELISA.

While the majority of these clones give signals that reveal some binding above background, in the present case, six clones gave very strong signals (See FIGURES 3a through 3e). DNA sequence analysis of these clones revealed 3 different light chain sequences. Two light chain sequences found in five out of six positive clones differ only in four amino acids, i.e., are 96 percent identical. The third light chain sequence shares about 80 percent identity with the other two. However, this sequence had two parts, each of which could be aligned to germ-lines of different V_κ families, and, thus is deemed likely to have arisen from PCR cross-over, an artifact that has been reported to occur frequently in the amplification antibody sequences.

Referring to FIGURES 3a through 3e, analysis of the six human V_κ revealed two groups of highly related κ chain sequences. In addition, the CDR1 loops of all six selected human V_κ, which are believed to play a role in the assembly of V_κ and V_λ, resemble the corresponding region of LM609 V_κ. This indicates that the template V_λ of LM609 together with the LM609 antigen, the human integrin $\alpha_V\beta_3$, selected for human κ chains that are related to the LM609 κ chain. The fact

that no repeated sequences were found may indicate that the grafted LCDR3 loop of LM609, which is identical in all selected human κ chains, is mainly responsible for the contribution of the LM609 κ chain to antigen binding.

This supposition is supported by two additional observations. First, the initial humanization approach was based on the original human κ chain libraries. Four rounds of panning selected a repeated human κ chain with a sequence related to the LM609 κ chain. However, the corresponding hybrid Fab appeared to bind only weakly to human integrin $\alpha_v\beta_3$. Therefore, the LCDR3 loop of LM609 was grafted in the human κ chain libraries, and, though only roughly estimated from ELISA, the binding of the corresponding selected hybrid Fab to human integrin $\alpha_v\beta_3$ improved.

Second, the selected phage were selected by two further rounds of panning against immobilized human integrin $\alpha_v\beta_3$. Again, soluble ones derived from selected phagemids were analyzed for binding to immobilized human integrin $\alpha_v\beta_3$ by ELISA. This time, all of the analyzed 20 clones were found to bind specifically. However, sequencing of 16 clones revealed no repeated sequences. It appears, thus, that a number of different human κ chain sequences that contain the LCDR3 loop of LM609 can substitute the LM609 κ chain without much difference in binding to human integrin $\alpha_v\beta_3$. This finding is believed to be of importance for the therapeutic application of humanized LM609.

Due to allotypic sequence variability, humanized antibodies can be neutralized by the patient's immune system after repeated injections. This problem is avoided by using humanized antibodies with identical

antigen binding properties but different amino acid sequences for repeated administrations.

As with the original LM609 light chain, the selected light chains are each κ light chains. Moreover, database screening revealed that they were derived from the same germ-line, namely DPK-26, belonging to the $V_κ6$ family. This speaks in favor of a strong selection towards these sequences because the $V_κ6$ family is not frequently found in human antibodies. An obvious reason for this strong selection is a relatively high sequence similarity of the selected human light chains with the original mouse light chain.

Referring to FIGURE 4, for comparison, four clones from the un-selected library were picked randomly and their light chain sequences determined. Three selected human light chains used in the comparison consist of eleven LCDR1 amino acids, the length of the original mouse LCDR1, while only one out of four unselected human light chains shared the same LCDR1 length with the original mouse sequence (see FIGURE 4).

Moreover, both LCDR1 and LCDR2 of the selected human light chains are highly similar to the corresponding mouse sequence. The C-terminal amino acid of framework region 2 of the original mouse light chain sequence, a lysine (Lys) shown in brackets in FIGURE 4, is an unusual amino acid at this position and, thus may be involved in the formation of the antigen binding site. Interestingly, all the selected human light chain sequences contain a lysine at this position, while all the unselected sequences contain a tyrosine (Tyr) instead. As a matter of fact, the $V_κ6$ family is the only human $V_κ$ family that contains a lysine at that position.

Taken together, this evidence shows that the mouse V_H template and the antigen selected for unbiased human V_K sequences are related to the original mouse V_K sequence. Three clones from the light chain selection, revealing weaker binding to integrin $\alpha_V\beta_3$ than the six clones discussed above but still having significant binding above background, were also analyzed by DNA sequencing. These analyses revealed three unrelated V_λ sequences, together with selected V_K sequences—except the one that stemmed from the PCR cross-over artefact—were used as templates in the humanization of the heavy chain of LM609.

Based on the aforedescribed humanization strategy, five humanized versions of the anti-human integrin $\alpha_V\beta_3$ monoclonal antibody LM609 were generated. The five version were revealed through the sequence analysis of 14 humanized clones that bind to $\alpha_V\beta_3$. Referring to FIGURES 8a and 8b, the amino acid sequences of a mouse V_L (SEQ ID NO:45) are compared to the amino acid sequences of the version A, and the combined versions (or groups) B, C, D, and E.

Similarly FIGURES 8c through 8e compare the amino acid sequences of a mouse V_H (SEQ ID NO:56) to the amino acid sequences of the five versions (or groups) A-E. Four of these versions, represented by clones 7, 8, and 22 (group B); 4, 31, and 36 (group C); 24, 34, and 40 (group D); and 2 (group E), are highly related in amino acid sequence. The sequence group BCDE in FIGURES 8a and 8b represent the four versions that share an identical V_L domain (SEQ ID NO:49). The amino acid sequence identity of their V_H domains (SEQ ID NOS:50-53), which are all derived from the germ-line DP-65 or the highly related DP-78, is at least 90% for each version.

In contrast, version five includes clones 10, 11, and 37 (A), and represents a humanized version with a V_H domain (SEQ ID NO:54) that is derived from a different germ-line family. This humanized version also contains a different V_L domain (SEQ ID NO:55), which is, however, 95% identical and derived from the same germ-line. Germ-lines were determined by nucleic acid sequence alignment using DNAPLOT software provided by the VBASE Directory of Human V Gene Sequences from the MRC Centre for Protein Engineering.

By preserving the original complementarity determining region sequences such as the LCDR3 (SEQ ID NO:2) and HCDR3 (SEQ ID NO:1) sequences of LM609, the disclosed humanization strategy ensures epitope conservation. Epitope conservation is a critical demand in the humanization of antibodies, especially in the case of LM609. The function-blocking anti-human integrin $\alpha_v\beta_3$ mouse monoclonal antibody LM609 binds to a yet unidentified nonlinear epitope that involves both the α_v and β_3 polypeptide chains. Importantly, by binding to this epitope LM609 induces apoptosis in $\alpha_v\beta_3$ expressing vascular cells, a unique feature among a number of anti-human integrin $\alpha_v\beta_3$ mouse monoclonal antibodies. LM609 does not recognize the related human integrin $\alpha_{IIb}\beta_3$. Any cross-reactivity with human $\alpha_{IIb}\beta_3$, which is expressed on platelets, precludes the use of LM609 as a tool in cancer therapy.

The five humanized versions of LM609, clones 2, 4, 7, 11, and 24, which had been selected by binding to immobilized, thus potentially denatured human integrin $\alpha_v\beta_3$, were tested for binding to native human integrin $\alpha_v\beta_3$ expressed on the cell surface. For this, binding of humanized LM609 to untransfected CS-1 hamster cells and CS-1 hamster cells transfected with either

human β_3 or β_5 cDNA was analyzed by flow cytometry. Like mouse LM609, but in contrast to a control antibody, all five humanized versions of LM609 revealed specific binding to CS-1 hamster cells transfected with human β_3 cDNA (See FIGURES 5a-5f).

Potential cross-reactivity of humanized LM609 with human integrin $\alpha_{IIb}\beta_3$ was analyzed by ELISA. While antibody Fab-9 with known cross-reactivity bound to both immobilized human integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, cross-reactivity was detected neither for mouse LM609 nor its five humanized versions (See FIGURE 6).

Thus, all evidence speaks in favor of epitope conservation through the process of humanization of LM609.

Affinity maturation is a highly relevant step in engineering antibodies for therapeutic applications. By increasing the target affinity, the *in vivo* concentration of an antibody that must be reached to be effective for therapy is lowered. In addition to reducing the costs of antibody therapy, low effective *in vivo* concentrations will help to reduce the chance of immune response.

The CDR walking strategy for the affinity maturation of antibodies has been described elsewhere, and is known in the relevant art. For the affinity maturation of humanized LM609 a sequential optimization of LCDR3 and HCDR3 was chosen (See FIGURE 7). The randomized region in both CDRs was confined to a stretch of four amino acids that revealed highest variability in human antibody sequences. *See* Kabat, E.A. et al. (1991) SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, U.S. Dept of Health and Human Services, Washington, D.C. Using NNK doping (Barbas, C.F. et al., (1994) *IN VITRO* EVOLUTION OF A NEUTRALIZING HUMAN ANTIBODY TO HIV-1 TO ENHANCE AFFINITY AND

BROADEN STRAIN CROSS-REACTIVITY, *Proc. Natl. Acad. Sci. USA* **91**, 3809-3813) the randomization of four codons results in 32^4 or $1x10^6$ different nucleotide sequences. Based on this and assuming a Poisson distribution (Clackson, T., and Wells, J.A. (1994) *IN VITRO* SELECTION FROM PROTEIN AND PEPTIDE LIBRARIES, *Trends in Biotechnol.* **12**, 173-184), $5x10^6$ independent transformants are required for a complete library with 99 percent confidence. For each of the five humanized versions of LM609 independent LCDR3 randomized libraries were generated. The number of independent transformants in each library lay well above $5x10^6$ (see Experimental Procedures, below). Ten completely different LCDR3 sequences were obtained when two clones of each of the five libraries were analyzed by DNA sequencing. The three libraries that were based on humanized LM609 clones 2, 4, and 24, which contain identical light chains and highly related heavy chains derived from germ-line DP-65, were pooled. Corresponding to their underlying germ-line sequences the three remaining libraries were named DPK-26ranLCDR3/DP-10, DPK-26ranLCDR3/DP-65, and DPK-26ranLCDR3/DP-78. These three libraries were selected in parallel.

To improve the selection of higher affinity LCDR3 mutants, a solid phase off-rate selection (Yang, W.P. et al. (1995) CDR WALKING MUTAGENESIS FOR THE AFFINITY MATURATION OF A POTENT HUMAN ANTI-HIV-1 ANTIBODY INTO THE PICOMOLAR RANGE, *J.Mol Biol.* **254**, 392-403) was used. In five subsequent cycles of selection 20 µg LM609 IgG was added to the well with 200 or 50 ng immobilized human integrin $\alpha_v\beta_3$ following phage incubation and washing. After 24 hours at room temperature, the well was washed again and bound phage eluted with trypsin. This off-

rate selection step is discussed in the following paragraph.

Protein interactions are characterized by thermodynamic and kinetic parameters. While the affinity constant ($K_a = k_{on}/k_{off}$) is an equilibrium constant, the association (k_{on}) and dissociation (k_{off}) rate constants are more relevant to *in vivo* processes which are beyond equilibrium. *See* Williams, A.F. (1991) OUT OF EQUILIBRIUM, *Nature* **352**, 473-474. In fact, to occur *in vivo*, interactions with high affinity, i.e., high K_a values, still rely on rapid association, i.e., high k_{on} values. Antibodies are subject to kinetic selection based on binding target antigens rapidly, in parallel with thermodynamic selection for high affinity binding in order to allow sufficient time for antigen clearance. *See* Foote, J., and Milstein, C. (1991) KINETIC MATURATION OF AN IMMUNE RESPONSE, *Nature* **352**, 530-532. A typical antibody/antigen interaction with a K_a value in the range of 10^9 M^{-1} associates rapidly with a k_{on} value in the range of 10^5 to 10^6 $M^{-1}s^{-1}$ and dissociates slowly with a k_{off} value in the range of 10^{-3} to 10^{-4} s^{-1}. An off-rate selection for affinity maturation, i.e., decreasing k_{off}, requires consideration of the half-life of the antibody/antigen interactions that is given by $t_{½} = \ln 2/k_{off}$. An antibody/antigen interaction with $k_{off} = 1x10^{-4}$ s^{-1} has a half-life of about 2 hours. A tenfold lower dissociation constant, i.e., $k_{off} = 1x10^{-5}$ s^{-1}, results in a tenfold longer half-life, i.e., about 20 hours. These long half-lives limit the off-rate selection in our affinity maturation protocol. Using a reasonable time frame, antibodies with dissociation constants below $1x10^{-6}$ s^{-1} can not be enriched even after multiple selection cycles. However, using a similar protocol, an antibody was selected against gp120 with a

k_{off} value in the range of 10^{-6} s^{-1}. The corresponding affinity constant was in the range of 10^{11} M^{-1}, a more than 400-fold improvement of the parental antibody.

Eight clones from each of the three independently selected libraries were analyzed by DNA sequencing. The LCDR3 sequences are shown in Table 1, below.

TABLE 1

Selected LCDR3 Mutants

Kabat position[1]	91	92	93	94
LM609	Ser	Asn	Ser	Trp
Library DPK-26ranLCDR3/DP-10[2]	Ser	Gln	Trp	Trp
	Ser	Gln	Trp	Trp
	Ser	Gln	Trp	Trp
	Ser	Gln	Trp	Trp
	Ser	Gln	Val	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	His
	Ser	Gln	Phe	His
Library DPK-26ranLCDR3/DP-65[3]	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
Library DPK-26ranLCDR3/DP-78[4]	Ser	Gln	Trp	Trp
	Ser	Gln	Trp	Trp
	Ser	Gln	Val	Trp
	Ser	Gln	Val	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Phe	Trp
	Ser	Gln	Tyr	Trp
	Ser	Gln	His	Trp

[1] cf. Kabat, et al. (1991)
[2] based on humanized LM609 clone 11
[3] based on humanized LM609 clones 2, 4, and 24
[4] based on humanized LM609 clone 7

A strong selection towards a consensus sequence that is highly related to the original sequence took place. All 24 analyzed clones contain a serine (Ser) in Position 91 and a glutamine (Gln) in Position 92 of the randomized region. The corresponding amino acids in the parental LCDR3 are serine (Ser) and asparagine (Asn), respectively. Interestingly, all three serine codons of the NNK genetic code (TCT, TCG, and ACT) are found in Position 91. Position 94, a tryptophane (Trp) in the parental LCDR3, was re-selected in 22 out of 24 clones. Two clones contain a histidine (His) instead. Only Position 93 reveals greater diversity. The original serine (Ser) is substituted by an aromatic or hydrophobic amino acid, either phenylalanine (Phe-13/24), tryptophane (Trp-6/24), valine (Val-3/24), tyrosine (Tyr-1/24) or histidine (His-1/24). Analysis of the heavy chain sequences revealed that no cross-contamination between the three independently selected libraries took place. All eight clones selected from library DPK-26ranLCDR3/DP-65, which contained the pool of the three highly related heavy chains encoding sequences derived from germ-line DP-65, were identical and derived from humanized LM609 clone 24.

The conserved LCDR3 sequence speaks in favor of a highly defined epitope on human integrin $\alpha_v\beta_3$. Though binding to native human integrin $\alpha_v\beta_3$ on the cell surface needs to be proved yet, an epitope shift towards denatured human integrin $\alpha_v\beta_3$ is unlikely. The selected phage pools were analyzed for binding to human integrin $\alpha_v\beta_3$ by phage ELISA and in competition with LM609 IgG. These analyses suggest a significantly lower dissociation constant of the selected clones in comparison with LM609 as well as humanized LM609. The

substitution of the original serine in Position 3 by an aromatic residue may give rise to a new hydrophobic interaction with a strong impact on the overall affinity.

Experimental Procedures

Materials

LM609 IgG purified from hybridoma cultures was provided by Dr. David A. Cheresh. Human integrin $\alpha_v\beta_3$ and human integrin $\alpha_{IIb}\beta_3$ were from sources described in HIGH-AFFINITY SELF-REACTIVE HUMAN ANTIBODIES BY DESIGN AND SELECTION: TARGETING THE INTEGRIN LIGAND BINDING SITE, Barbas III, C.F., et al. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 10003-10007. Untransfected CS-1 hamster cells and CS-1 hamster cells transfected with either human β_3 or β_5 cDNA were obtained from Dr. David A. Cheresh (Filardo, E.J., et al. (1995) REQUIREMENT OF THE NPXY MOTIF IN THE INTEGRIN β_3 SUBUNIT CYTOPLASMIC TAIL FOR MELANOMA CELL MIGRATION *IN VITRO* AND *IN VIVO*, *J.Cell Biol.* **130**, 441-450) and maintained in RPMI medium supplemented with 5% fetal calf serum (FCS) at 37°C in 7% CO_2.

E.coli expression of soluble humanized LM609 Fab

Following phage library panning, the SfiI insert of the selected humanized LM609 phagemid pool was cloned into the *E.coli* expression vector pPhoA-H6HA (*See* Rader, C., and Barbas III, C.F. (1997) PHAGE DISPLAY OF COMBINATORIAL ANTIBODY LIBRARIES, *Curr. Opin. Biotechnol.* **8**, 503-508) for detection of $\alpha_v\beta_3$ binders. Sequence determination of 14 clones revealed five different humanized LM609 versions, represented by clones 2, 4, 7, 11, and 24. cDNAs of these clones were cut out by SacI/SpeI digestion and ligated into SacI/NheI cut pComb3H, thereby removing the gene III fragment encoding

cDNA of pComb3H and allowing for production of soluble Fab (*See* Rader, C. (1997) *Curr. Opin. Biotechnol.* **8**, 503-508). The ligation products were electrotrasformed into *E.coli* strain XL1-Blue. Fab production was induced by addition of isopropyl β-D-thiogalactopyransoside as described (Barbas III, C.F., et al. (1991) ASSEMBLY OF COMBINATORIAL ANTIBODY LIBRARIES ON PHAGE SURFACES: THE GENE III SITE, *Proc. Natl. Acad. Sci. USA* **88**, 7978-7982).

ELISA

Human integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ were coated for 90 minutes at 37°C on a 96-well plate (Costar #3690) at a concentration of 60 ng/25 µl metal buffer (25 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM $MnCl_2$) per well. Following blocking with 150 µl 3% BSA/TBS for 1 hour at 37°C, 25 µl crude supernatants from overnight cultures of *E.coli* strain XL1-Blue expressing soluble LM609 or humanized LM609 Fab were added to the well and incubated for 2 hours at 37°C. Binding of each of the supernatants to wells coated with human integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ as well as to uncoated but blocked wells was analyzed in triplicates. As a positive control, 25 µl of 50 ng/µl purified Fab-9, an antibody binding to both human integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (Barbas III, et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10003-10007), and as a negative control, 25 µl of plain bacterial culture medium were used. After extensive washing with tap water, 25 µl of a 1:2,000 dilution of goat anti-mouse $F(ab')_2$ or goat anti-human $F(ab')_2$ conjugated with alkaline phosphatase (Pierce #31324 or 31312, respectively) in 1% BSA/TBS was added to the well and incubated for 1 hour at 37°C. Following another extensive washing with tap water, 50 µl of alkaline phosphatase substrate (5 mg disodium p-

nitrophenyl phosphate hexahydrate (Sigma #104-105) dissolved in 5 ml 10% diethanolamine, 1 mM $MgCl_2$, 3 mM NaN_3, pH 9.8) were added to the well. The plate was analyzed with an ELISA reader (Molecular Devices) after 15 minutes incubation at room temperature.

Flow cytometry

Flow cytometry was performed using a Becton Dickinson FACScan instrument. For each determination, $5x10^3$ untransfected hamster CS-1 cells or hamster CS-1 cells expressing either human β_3 or β_5, were analyzed. Indirect immunofluorescence staining was achieved with crude lysates of *E.coli* strain XL1-Blue expressing soluble humanized LM609 Fab or, as a negative control, an unrelated human Fab. A 1:100 dilution of FITC-conjugated goat anti-human $F(ab')_2$ (Jackson #109-096-097) was used for detection.

Construction of LCDR3 libraries

Humanized LM609 clones 2, 4, 7, 11, and 24 in pPhoA-H6HA were separately utilized as templates for overlap extension PCR mutagenesis as described (Barbas III, et al. (1994) *IN VITRO* EVOLUTION OF A NEUTRALIZING HUMAN ANTIBODY TO HIV-1 TO ENHANCE AFFINITY AND BROADEN STRAIN CROSS-REACTIVITY, *Proc. Natl. Acad. Sci. USA* **91**, 3809-3813). The two fragments required for this procedure were obtained with the PCR primer pairs OMPSEQ (SEQ ID NO:39) / CR320 (SEQ ID NO:46) and CR520 (SEQ ID NO:47) / DPSEQ (SEQ ID NO:48), respectively. The resulting five cDNAs with randomized LCDR3 were cut with SfiI, ligated into the appropriately digested phagemid vector pComb3H, and electrotransformed into *E.coli* strain ER 2537. Two clones of each of the five libraries were analyzed by DNA sequencing and revealed correct assembly as well as

10 different LCDR3 sequences. Prior to selection, libraries based on clones 2, 4, and 24 (V_L germ-line DP-26; V_H germ-line DP-65) were combined to give a complexity of $6x10^7$ independent transformants. Libraries based on clone 11 (V_L germ-line DP-26; V_H germ-line DP-10) and 7 (V_L germ-line DP-26; V_H germ-line DP-78) were kept separate with a complexity of $3x10^7$ and $4x10^7$ independent transformants, respectively.

Selection of LCDR3 libraries

The three LCDR3 libraries were separately selected by panning against immobilized integrin $\alpha_v\beta_3$ for six cycles. Panning was performed substantially as described hereinabove for the LM609 humanization. The concentration of human integrin $\alpha_v\beta_3$ for coating was 200 ng/25 µl in the first through fourth cycles and 50 ng/25 µl in the fifth and sixth cycles. Also, the input number of phage, in the range of 10^{12} in the first through fourth cycles as usual, was decreased by a factor of 10 in the fifth cycle and by a factor of 100 in the sixth cycle. In the second through the sixth cycles of selection 20 µg LM609 IgG in 50µl metal buffer (25 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM $MnCl_2$) was added to the well following removal of unbound phage by five to ten washing steps. The plate was then incubated for 24 hours at room temperature (off-rate selection) prior to five additional washing steps and trypsin elution as described. After the sixth cycle phage were produced from single clones and tested for binding to immobilized human integrin $\alpha_v\beta_3$ by phage ELISA using a sheep anti-M13 antibody conjugated with horseradish peroxidase (Pharmacia #27-9411-01) as a secondary antibody. Light chain and heavy chain encoding sequences of positive

clones were analyzed by DNA sequencing using the primers OMPSEQ (SEQ ID NO:39) and PELSEQ (SEQ ID NO:24), respectively.

Optimization of LM609 by CDR randomization

In addition to the humanization, LM609 can be optimized in two respects: First, by increasing the affinity to $\alpha_v\beta_3$ and, second, by broadening the species cross-reactivity. Increased affinity of engineered LM609 increases the potency and decreases the cost of a potential cancer therapy.

The original mouse monoclonal antibody LM609 already has a broad species cross-reactivity. It binds to human, dog, cat, bovine, rabbit, and chick but not mouse $\alpha_v\beta_3$. The fact that LM609 does not recognize host $\alpha_v\beta_3$ in the mouse models of human cancer is a major concern for the therapeutic applicability of LM609. Engineered LM609 binding to both human and mouse $\alpha_v\beta_3$ would be an important tool towards clinical trials. *In vitro* methods for the improvement of monoclonal antibody affinity include *chain shuffling* (*See* Marks, J.D., et al. (1992) BY-PASSING IMMUNIZATION: BUILDING HIGH HUMAN ANTIBODIES BY CHAIN SHUFFLING, *Bio/Technology* **10**, 779-783). Binding to $\alpha_v\beta_3$ can be further improved by subsequent CDR randomization, an approach termed *CDR walking* (*See* Barbas III, C.F., et al. (1994) *IN VITRO* EVOLUTION OF A NEUTRALIZING HUMAN ANTIBODY TO HIV-1 TO ENHANCE AFFINITY AND BROADEN STRAIN CROSS-REACTIVITY, *Proc. Natl. Acad. Sci. USA* **91**, 3809-3813). The *in vitro* strategies for humanization and affinity improvement of LM609 are likely to generate cross-reactivity with mouse $\alpha_v\beta_3$ concurrently. Directed selection for mouse $\alpha_v\beta_3$ recognition is complicated by the fact that mouse $\alpha_v\beta_3$ has not been purified yet. However, several mouse cell

lines, e.g., NIH/3T3, are known to express $\alpha_v\beta_3$ and, thus, may be included in the screening procedure.

The foregoing discussion and the accompanying examples are presented as illustrative, and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.